EXHIBIT A

Center For Blood Research
Report of Invention

CONFIDENTIAL

Case Number: _____

Title of Invention: CCR5 siRNA mediated sustained inhibition of HIV infection

Investigator(s):

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Grants, Gifts or Contracts, which supported the work leading to this invention (include all private, federal and non-federal funding):

	Sponsor	Award number	Time period
1.	NIH	5921	
2.	NIH	5101	
3.	NIH	5603	

Lab or Department Where Developed: Shankar Lab

Description of Invention (please type or print clearly):

1. Brief Summary:
   We designed an siRNA duplex targeting CCR5 gene, which resulted in long-term reduction of CCR5 expresion on macrophages. Moreover, silencing of CCR5 expression provided sustained protection against HIV-1 replication in macrophages, which are major targets of the virus.
2. Background/Improvement over prior art (please include specific literature references, if known):
   CCR5 is a chemokine receptor on macrophage cell surface important for HIV-1 entry, but dispensable for normal immunity. Commercially available drugs, which inhibit CCR5 expression to prevent HIV viral entry, such as RANTES, are mostly chemokines that induce inflammatory reaction. RNA interference is a novel powerful weapon that eliminates gene expression.

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3. Detailed Description (add pages, figures, drawings, etc., as needed, as well as any relevant manuscripts or articles):
See attached manuscript.

4. Expected uses and /or commercial applications (list as many as you feel are feasible):

(1) CCR5 siRNA duplexes may be delivered directed in circulation to inhibit the expression of CCR5 on CD4 T cells or macrophages, and thus help to prevent the entry of HIV-1 virus.
(2) CCR5 siRNA may be engineered for expression in bone marrow derived stem cells to generate HIV resistant immune cells.
(3) CCR5 silencing may also be applicable in organ transplantation to eliminate inflammatory infiltration in allografts, and thus prevent the onset of acute or chronic rejection.

First Written Disclosure: Date: _____ Witnessed by: _____
Form of disclosure (journal article, thesis, oral presentation, web posting, etc.)

First Reduction to Practice: Date _____ Witnessed by: _____

Supporting Reports, Lab Records, Data, etc. (include dates and locations):
Manuscript attached

Contacts or agreements with commercial entities (include name of person contacted):
Premlata Shankar

Inventor		**Witness**	
Premlata Shankar	_____	_____	_____
(Signature)	date	(Signature)	date

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Abstract

Macrophages constitute an important reservoir in HIV-1 infection[1,2]. However, although the potential of short interfering RNAs (siRNAs) to suppress HIV-1 has recently been demonstrated in cell lines[3-5] and proliferating CD4 T cells[4,5], whether stable siRNA-mediated HIV suppression can be achieved in terminally differentiated macrophages is not known. We studied the anti-viral effect of siRNAs targeting CCR5, the major cellular co-receptor for M-tropic HIV and the viral structural gene, p24 in primary monocyte-derived macrophages (MDMs). Both siRNAs, introduced 2 days prior to viral infection, effectively inhibited HIV-1 replication for a much longer time than reported in cell lines[3,5] and combined targeting of both genes was able to abrogate viral infection. As the interval between transfection and infection was raised from 2-15 days, the HIV suppressive effect of viral p24 siRNA progressively decreased, and was eventually lost. In contrast, viral inhibition by cellular CCR5 knockdown was uniformly sustained even when infection preceded transfection by 15 days. However, post-infection delivery of p24 siRNA (into cells actively replicating virus) was able to inhibit viral replication by over 6 folds uniformly for up to 15 days after transfection. Blocking viral entry by targeting CCR5 post-infection, as expected had no effect on viral replication. In addition to demonstrating the feasibility of targeting cellular and viral genes in physiologically relevant cells as a potential preventive and a curative approach against HIV, these results also suggest that siRNAs need target mRNAs for their intracellular sustenance.

Synthetic 21-nucleotide siRNA duplexes, targeting several regions of the HIV-1 genome can suppress viral replication in human cell lines and activated CD4 T

cells[3,4,6,7]. However, the silencing effect siRNAs in these actively replicating cells peak around 96 h, but taper off thereafter and is completely lost by day 9[7], presumably because of siRNA dilution with cell division or degradation inside the cell[8]. Macrophages are terminally differentiated non-dividing cells that constitute a significant reservoir for HIV-1 in vivo[1,2]. They are also relatively immune to the cytopathic effects of HIV and survive for long periods after infection[9]. Thus, we investigated whether more sustained siRNA-mediated viral silencing could be achieved in MDMs.

Previous reports on siRNA-directed protection against HIV-1 have targeted viral genes[3,4,7] or used the cellular receptor, CD4[7]. However, CD4 targeting may not be a feasible approach because of its importance in immune functions. On the other hand, CCR5, the major HIV coreceptor for viral entry into macrophages, may be a better cellular target since a 32 bp homozygous deletion of the gene has no deleterious immunological consequences[10] but provides protection from HIV infection[11,12]. Thus, to determine anti-HIV effects of siRNA in macrophages, we selected CCR5 and the viral p24 gene. First, we verified that MDMs are susceptibile for siRNA delivery by transfection using Cy5-labeled p24 siRNA. After 24 h of transfection, 84% of CD14+ macrophages were Cy5+ by flow cytometry(Fig 1*a*), a transfection efficiency comparable to that observed with HeLa cells (90%, not shown). The siRNA was not taken up by non-specific phagocytosis because, in the absence of oligofectamine, < 6% of MDMs were Cy5+ (Fig. 1*a*). To determine anti-viral effects of siRNAs, MDMs were transfected with CCR5 or viral p24 siRNAs singly or in combination, and challenged with R5 (BaL) macrophage tropic virus 2 days later. Periodic measurement of cell-free viral particle production by p24 ELISA of culture supernatants from either CCR5 or p24 siRNA transfected MDMs revealed a 4-6 fold

reduction compared to mock and GFP siRNA-transfected controls (Fig. 1*b*). Similarly, flow cytometric analysis of p24 expression also demonstrated a 7 fold reduction in p24 expression with CCR5 or p24 siRNA transfection as compared to controls for up to 15 days (Fig. 1*c*). More importantly, transfection with both siRNAs was able to abrogate HIV infection throughout the 15- day period of observation (Fig. 1*b* and 1*c*). Combined treatment was more effective probably because of interruption of 2 steps in the viral life cycle, with CCR5 siRNA blocking viral entry and p24 siRNA destroying the virus slipping through the remaining coreceptors or taken up passively. Thus, siRNAs can provide lasting protection against HIV in macrophages.

Our results suggest a role for siRNA for potential preventive strategy. However, to realize this potential, it is important that siRNAs persist in the cells for long periods of time before infection. To address this issue, we first determined the longevity of transfected Cy5-labeled p24 siRNA and CCR5 siRNA-mediated suppression of endogenous CCR5 expression in uninfected MDMs. The levels of intracellular Cy5-p24 siRNA in uninfected MDMs, measured by flow cytometry, gradually declined with time and was undetectable 10 days after transfection (Fig. 2*a*). In contrast, CCR5 expression in CCR5 siRNA transfected cells remained uniformly silenced from 1-20 days after transfection (Fig. 2*b*). To test if these differences in intracellular survival are also reflected in their ability to suppress HIV, we transfected MDMs with CCR5 or p24 siRNA and initiated infections at increasing intervals after transfection and measured p24 levels 10 days after infection. Consistent with long term suppression of CCR5 expression, compared to control GFP siRNA, CCR5 siRNA was able to provide equivalent level of protection whether the cells were infected 2 or 15 days after transfection (Fig. 2*c*). On the other hand, p24 siRNA provided maximal protection when the cells were infected within 5 days after

transfection but showed a declining level of protection when the interval between transfection and infection was extended further. The reason for the differential longevity of siRNAs targeting cellular vs viral gene is not clear. Considering that MDMs are non-dividing cells, it is unlikely to be due to differential dilution during cell division. While the cellular genes are continuously transcribed, providing a substrate for siRNA, viral RNA will be available only after infection. The differential effects of targeting cellular and viral genes in non-dividing MDMs, together with the fact that Cy-labeled p24 RNA was only maintained up to 7 days in uninfected cells, prompt us to propose that continued presence of the substrate RNA may be needed for intracellular sustenance of siRNA. This hypothesis is further strengthened by our results that p24siRNA was able to suppress HIV for 15 days when infection was done 2 days after transfection (Fig. 1*c*). In fact, sustenance by self-propagation of siRNA by siRNA primed-RNA dependent RNA polymerase (RdRP) is well known in C. elegans and drosophila[13,14].

To determine the anti-viral potential of siRNAs in established infection, we infected MDMs for 16 days before transfecting with CCR5 or p24 siRNA. Prior to transfection, >90% of MDMs were infected (not shown). These cells were transfected with either CCR5 or p24 siRNA and the suppression of viral replication followed over time. As expected, CCR5 blockade did not significantly reduce virus replication in this setup (Fig. 3*a*). In striking contrast, p24 siRNA was able to reduce viral replication by >90% throughout the 15-day period of observation (Fig. 3*b*). These results contrast with the inability of p24siRNA to confer HIV resistance when transfection preceded infection by more than 5 days (Fig. 2*c*). In addition to providing evidence that long lasting viral suppression can be achieved in MDMs with

established infection, these results also support the idea that the siRNA effect is sustained in the continued presence of target mRNA.

Collectively, our results demonstrate for the first time the feasibility of RNAi-based therapeutics to achieve long lasting suppression of HIV-1 in a physiologic setup, both to prevent infection and to suppress replication in established infection. It is remarkable that a single application of synthetic siRNA was able to afford long lasting protection against HIV in MDMs. Macrophages represent a key target of HIV in vivo and although the absolute number of infected macrophages is relatively low compared to CD4 T cells, the unique dynamics of HIV replication in these cells makes them a formidable viral reservoir[15]. Macrophages are relatively immune to the cytopathic effects of HIV and can survive for long periods after infection. They replicate large amounts of virus in sequestered cytoplasmic vacuoles with a plateau of virus production lasting for as long as 60 days[9]. Thus, it is significant this recalcitrant reservoir cells are particularly amenable for siRNA-mediated viral inhibition.

Our results also raise the possibility that target mRNA may sustain or amplify the input siRNA. However, the siRNA effect rapidly fades in cell lines even in the presence of target mRNAs[16-18]. Whether siRNA dilution with cell division is able to overcome the possible benefits of target mRNAs or whether siRNAs operate differently in different cell types is not clear. These issues underscore the need to elucidate the fundamental mechanisms of RNAi in mammalian cells. Non-dividing MDMs might provide an ideal cell type for such mechanistic studies in the absence of dilution effect complicating analysis.

Drawbacks of the siRNA approach have also now started to surface. A single nucleotide substitution in the middle of the targeted sequence can lead to the emergence of mutant virus resistant to RNAi[19]. This underscores the importance of

targeting highly conserved regions of the viral genome as well as using combinations of siRNAs. Moreover, efficient delivery into CD4 T cells and macrophages remains the major bottleneck in gene therapeutic approaches to HIV. However, two recent studies have shown the feasibility of in vivo delivery by intravenous injection in mice[20,21]. Given the rapid strides being made in the field, the use of siRNA as an anti-viral therapeutic approach in humans appears to be promising.

Methods

Preparation of human MDMs. Human monocytes were isolated from buffy coats prepared from normal volunteer donors. Peripheral blood mononuclear cells were prepared by Ficoll-Hypaque (Pharmacia Corperation, Peapack, New Jersey) density gradient centrifugation and were seeded at 2×10^6 cells/ml in 24-well plates in RPMI 1640 medium (BioWhittaker, Inc., Walkersville, Maryland) supplemented with 10% heat-inactivated human AB serum (Nabi, Boca Raton, Florida), 50 U/ml penicillin, 50 μg/ml streptomycin and 2 mM L-glutamine. After 5 days of culture, non-adherent cells were removed by repeated gentle washings with warm medium, and the adherent cells were harvested by tripsinization. Over 95% of the adherent cells obtained with this technique were $CD14^+$ macrophages (not shown).

Preparation of siRNAs. All siRNAs, including the cy5-labeled p24 were synthesized at Dharmacon Research, Lafayette, Colorado. The sequences of sense and anti-sense strands of siRNAs were as followed: CCR5, 5'-P.CUCUGCUUCGGUGUCGAAAdTdT-3' (sense), 5'-P.UUUCGACACCGAAGCAGAGdTdT-3' (antisense); p24, 5'-

P.GAUUGUACUGAGAGACAGGCU-3' (sense), 5'-
P.CCUGUCUCUCUCAGUACAAUCUU-3' (antisense); GFP, 5'-
P.GGCUACGUCCAGGAGCGCACC-3' (sense), 5'-
P.UGCGCUCCUGGACGUAGCCUU-3' (antisense). The RNAs were deprotected and annealed according to the manufacturer's instruction.

Transfection of siRNAs. Macrophages were transfected with oligofectamine (Gibco-Invitrogen, Rockville, Maryland) according to the manufacturer's protocol in the presence or absence of 1 n mol siRNA duplex. After overnight incubation, the cells were washed and used for further study.

Flow cytometry. To test CCR5 expression and HIV-1 infection, MDMs were stained with biotin-conjugated αCCR5 antibody (R&D Systems, Inc., Minneapolis, Minnesota) followed by avidin-labled streptavidin-PE (BD Pharmingen, San Diego, California), or FITC-labeled αp24 Mab (Beckman Coulter, Brea, California) and analyzed in a flow analyzed on FACScalibur with CellQuest software (Becton Dickinson, Franklin Lakes, New Jersey).

HIV-1 infection. At various time points before or after siRNA transfection, macrophages were infected with R5 BAL strain of HIV-1 using 50 ng of p24 gag antigen per well. At indicated times, HIV-1 replication in infected macrophages was evaluated by flow cytometric analysis of p24 expression, and cell-free viral production was measured by ELISA for p24 antigen in supernatants using.....

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Legends

Fig. 1 CCR5 and p24 siRNAs prevent HIV_{BaL} infection in monocyte-derived macrophages. ***a,*** Flow-cytometric analysis of siRNA transfection efficiency 24 h after MDMs (CD14-positive cells) were untreated, transfected with or exposed to cy5-labeled p24 siRNA. Percentage of macrophages with cy5-labeled p24 siRNA was given in each panel. ***b,*** Dynamics of cell-free HIV_{BaL} viral particle production from mock-transfected (◆), control GFP siRNA- (■), p24 siRNA- (▲), CCR5 siRNA- (×), and p24+CCR5 siRNA-transfected (∗) macrophages as measured by ELISA for p24 antigen. Error bars are the average of 2 experiments. ***c,*** siRNA-directed inhibition of viral gene expression demonstrated by flow-cytometric analysis of p24 expression (p24-FITC) 15 days after HIV_{BaL} infection in MDMs mock-transfected or transfected with control GFP-, p24-, CCR5- and p24+CCR5-siRNAs 2 days prior to viral infection and in uninfected control. Percentage of gated p24-positive cells are shown in each panel.

Fig. 2 Longevity of siRNA and sustenance of its silencing effect in MDMs. ***a,*** Kinetics of cy5-labeled p24 siRNA in uninfected macrophages demonstrated by flow cytometric analysis up to 10 days after transfection. Percentage of macrophages with

cy5-labeled p24 siRNA was given in each panel. ***b,*** Time-dependent expression of endogenous CCR5 expression demonstrated by flow cytometric analysis after uninfected MDMs were transfected with CCR5 siRNA (upper panel, green) or control GFP siRNA (lower panel, green) in comparison to mock transfection (white). ***c,*** siRNA-directed inhibition of viral gene expression demonstrated by flow-cytometric analysis of p24 expression (p24-FITC) 10 days after HIV_{BaL} infection initiated at increasing intervals (shown on top of each column) after MDMs were transfected with control GFP-, p24-, and CCR5-siRNAs. Percentage of gated p24-positive cells are shown in each panel.

Fig. 3 siRNA-directed inhibition of HIV_{BaL} replication in MDMs with established infection. ***a,*** Flow-cytometric analysis of p24 expression (p24-FITC) at increasing intervals (shown on top of each panel) after MDMs with established HIV_{BaL} infection (16 days after infection) were mock-transfected or transfected with control GFP- and p24-siRNA. Percentage of gated p24-positive cells are shown in each panel. ***b,*** Viral replication in infected MDMs 3 days after mock-transfection or transfection with control GFP- and CCR5-siRNA, determined by flow-cytometric analysis of p24 expression (p24-FITC). Percentage of gated p24-positive cells are shown in each panel.

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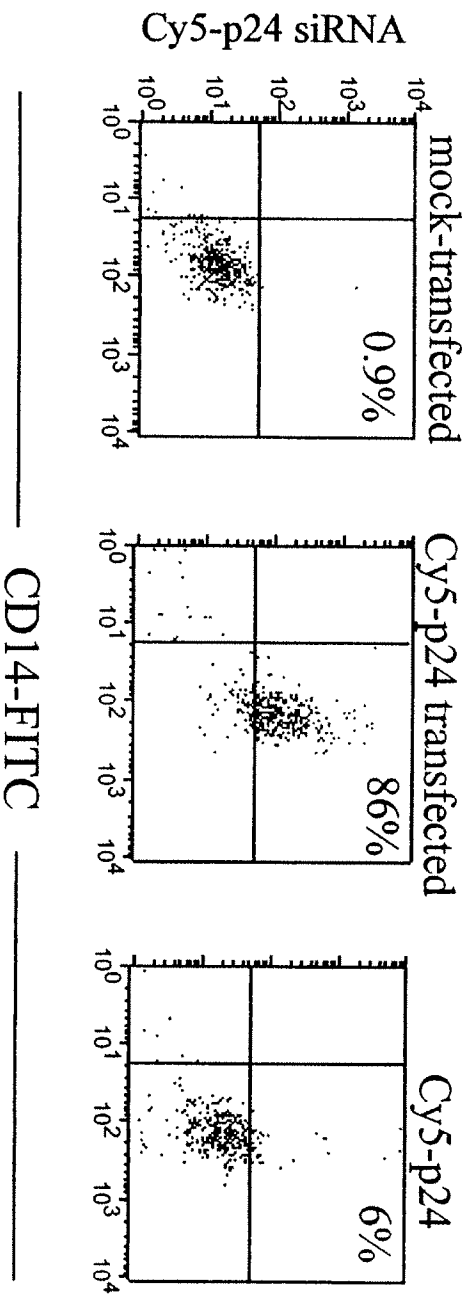

Fig.1a

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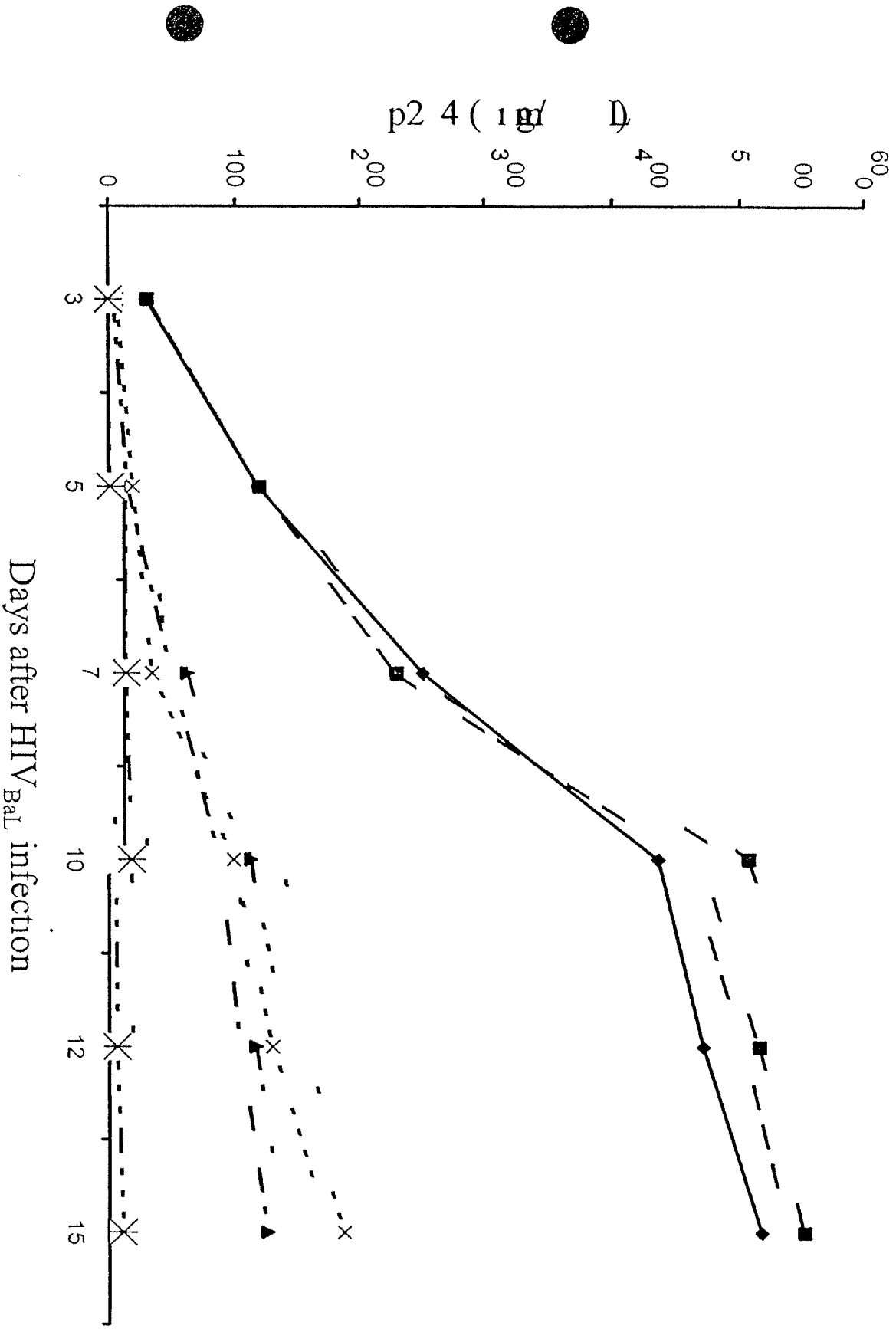
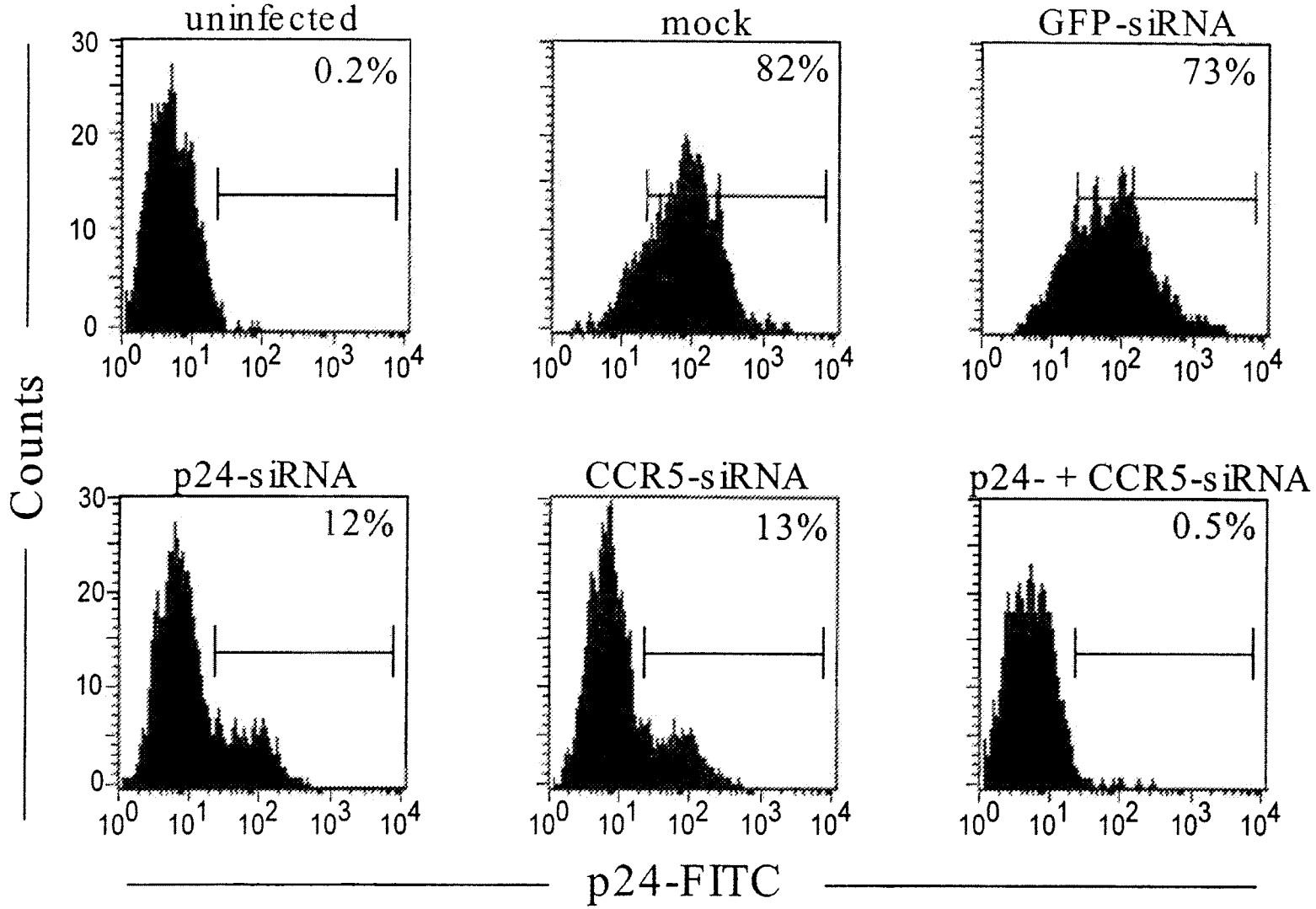


Fig. 1b

Fig. 1c



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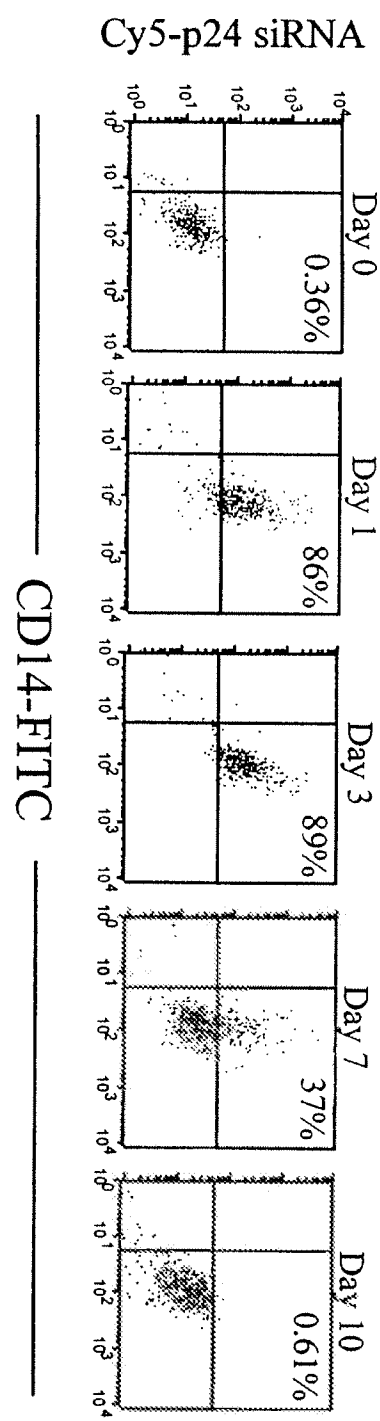

Fig. 2a

Fig. 2b

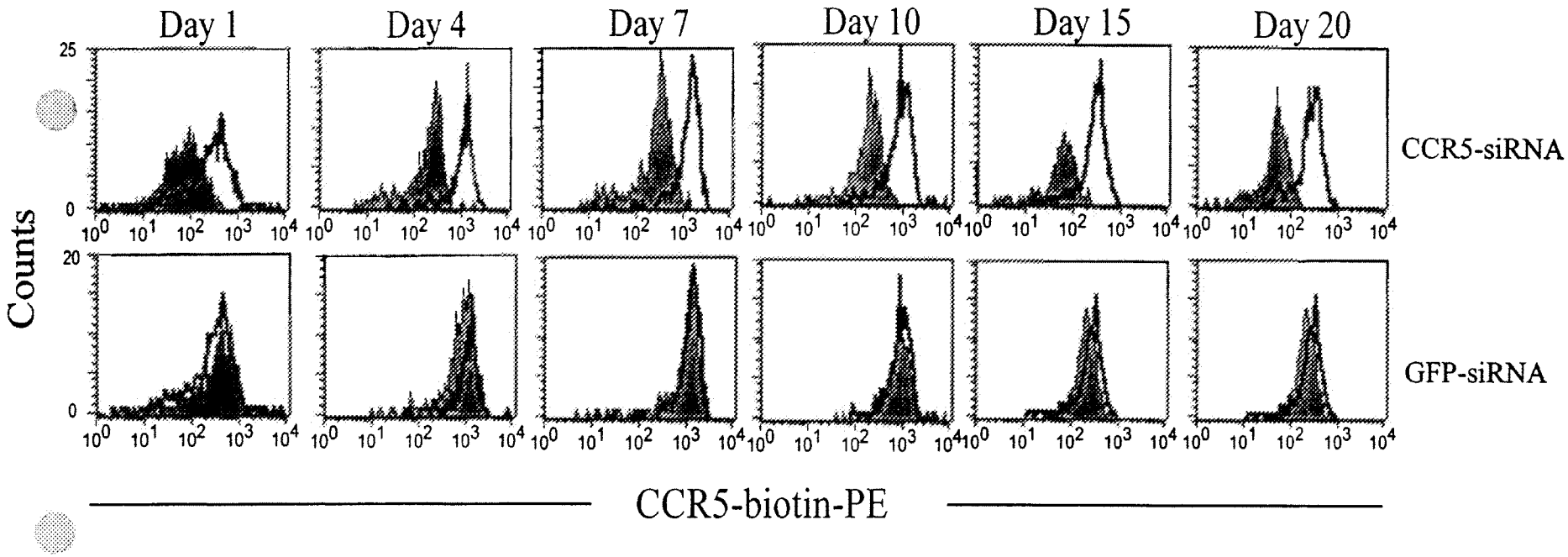

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Figure 2C

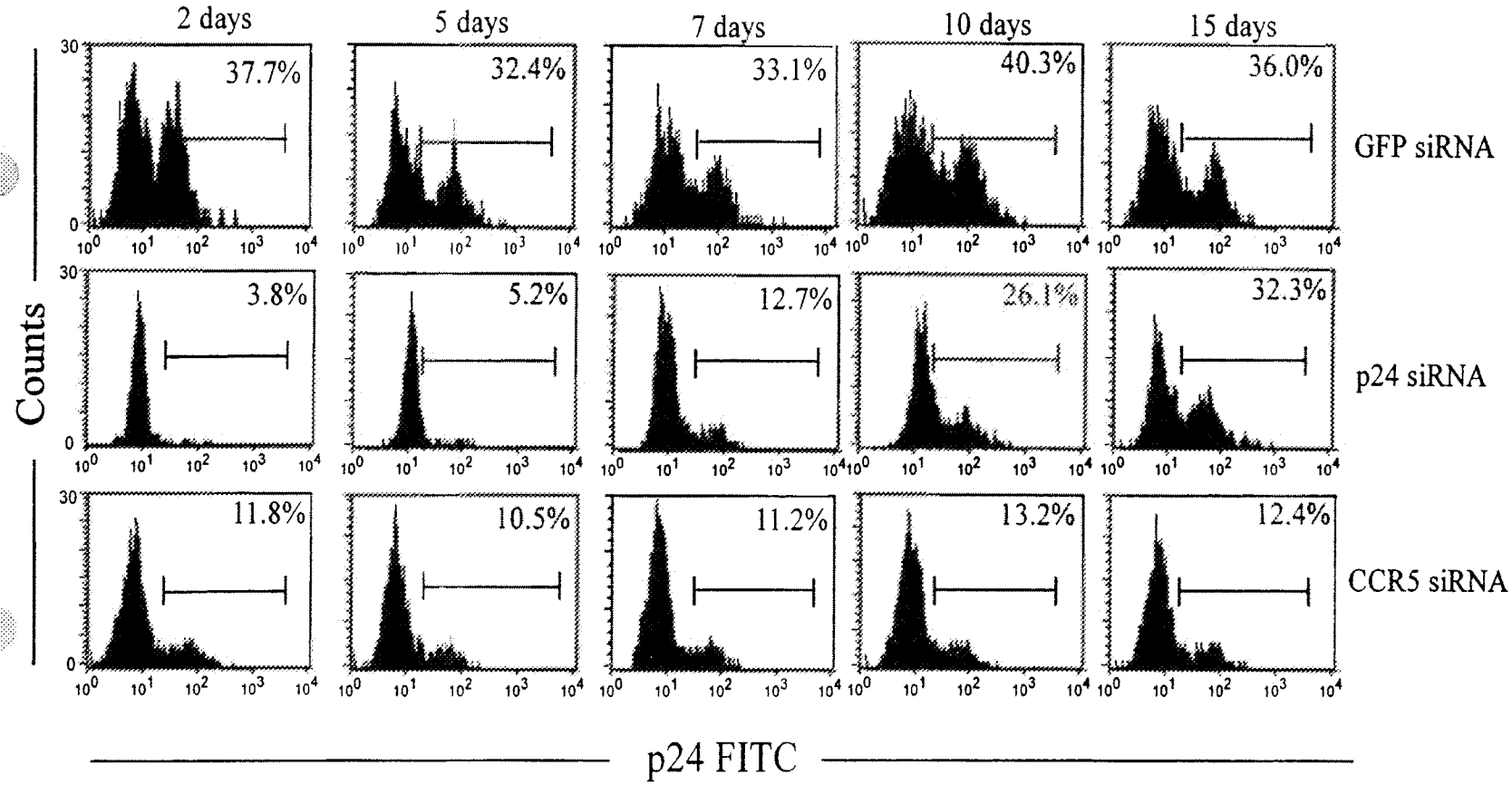

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Fig. 3A

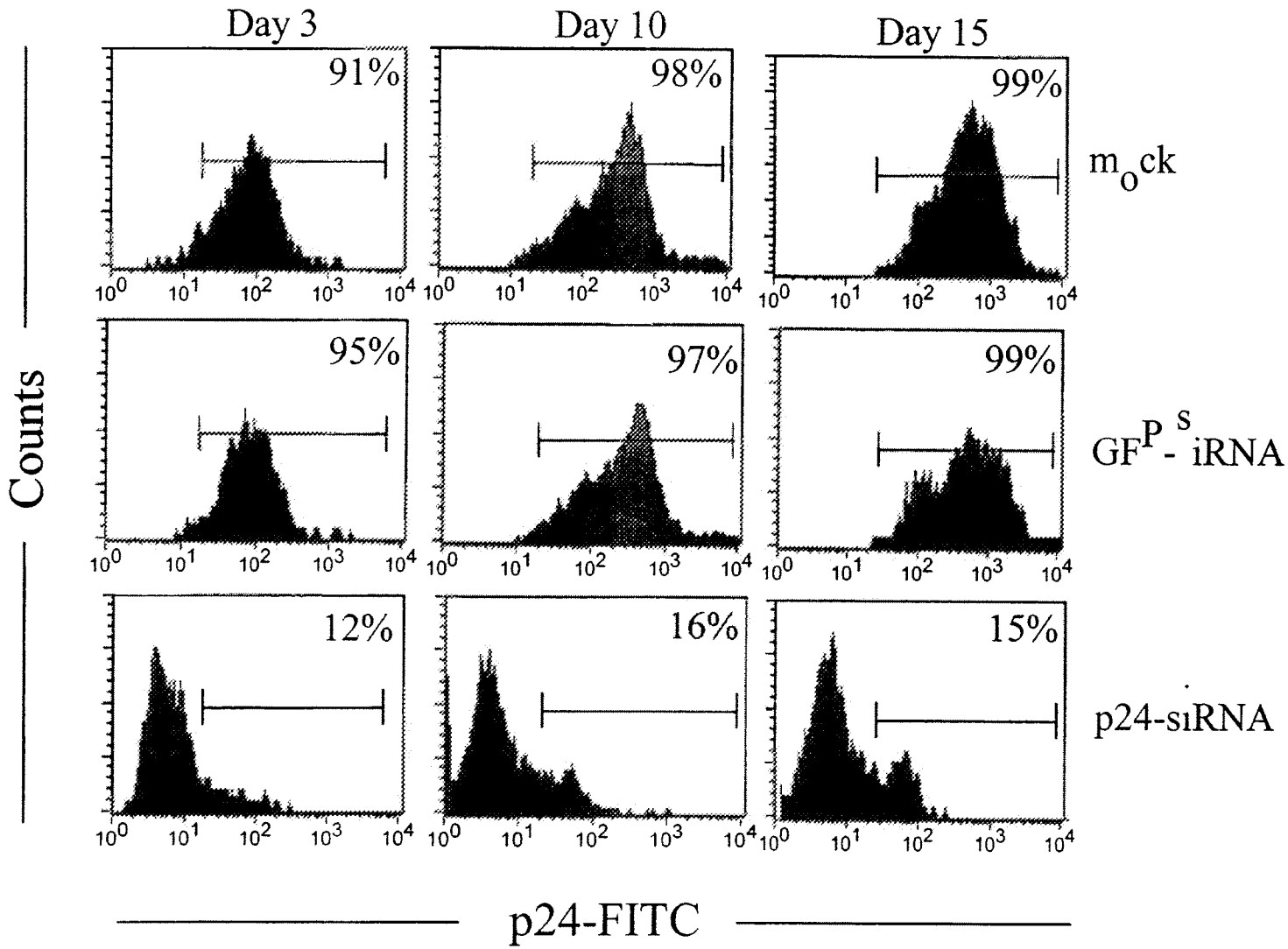

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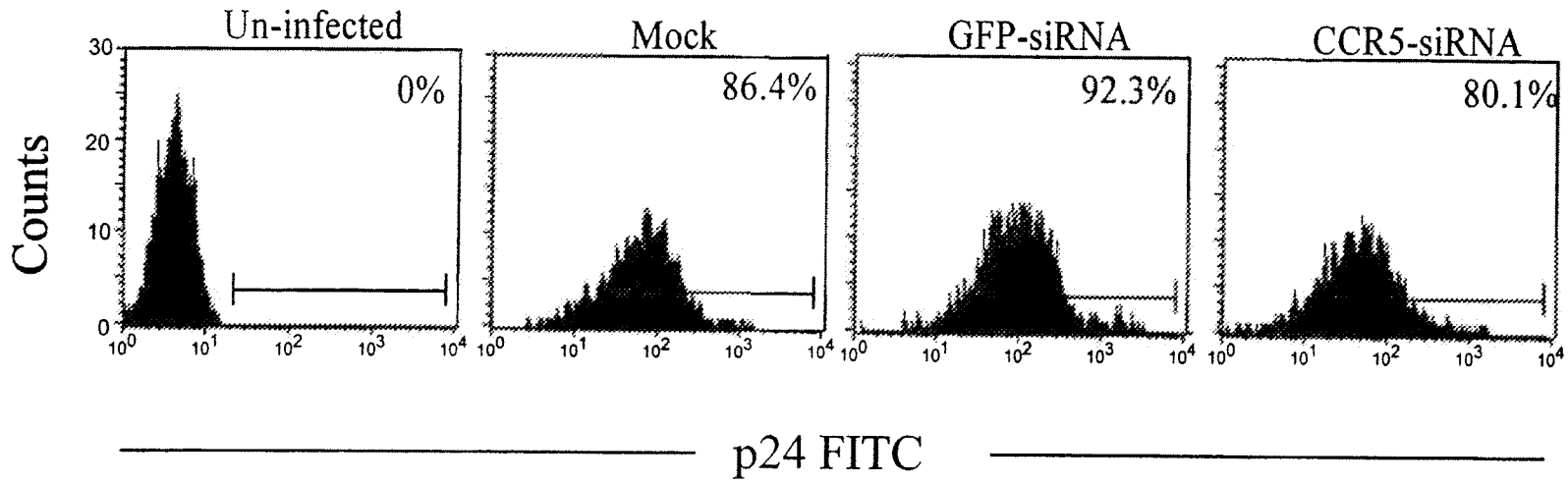
Figure 3B



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